Plantamajoside ameliorates lipopolysaccharide-induced acute lung injury via suppressing NF-κB and MAPK activation

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Despite developments in the knowledge and therapy of acute lung injury in recent decades, mortality remains high, and there is usually a lack of effective therapy. Plantamajoside, a major ingredient isolated from Plantago asiatica L. (Plantaginaceae), has been reported to have potent anti-inflammatory properties. However, the effect of plantamajoside on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice has not been investigated. The present study aimed to reveal the potential mechanism responsible for the anti-inflammatory effects of plantamajoside on LPS-induced acute lung injury in mice and in RAW264.7 cells. The results of histopathological changes as well as the lung wet-to-dry ratio and myeloperoxidase (MPO) activity showed that plantamajoside ameliorated the lung injury that was induced by LPS. qPCR and ELISA assays demonstrated that plantamajoside suppressed the production of IL-1β, IL-6 and TNF-α in a dose-dependent manner. TLR4 is an important sensor in LPS infection. Molecular studies showed that the expression of TLR4 was inhibited by plantamajoside administration. Further study was conducted on nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) using pathways using western blots. The results showed that plantamajoside inhibited the phosphorylation of p-38, p44/p42, JNK and ERK. All results indicated that plantamajoside has protective effect on LPS-induced ALI in mice and in RAW264.7 cells. Thus, plantamajoside may be a potential therapy for the treatment of pulmonary inflammation.

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1. Introduction

Acute lung injury (ALI) is characterized by hypoxemia, disruption of the alveolar-capillary membrane barrier, and pulmonary edema [1]. Its severe form, acute respiratory distress syndrome (ARDS), often leads to multiple organ failure with a mortality of approximately 30–50% [2]. Although some novel therapeutic strategies, such as the candidate gene approach [3] and mesenchymal stem cells [4], have been applied in ALI, the mortality rate remains high [5]. LPS, a major constituent of the Gram-negative bacterial cell wall, has been widely used to induce ALI in a mouse model [6–8].

It is well known that LPS plays a vital role in inflammatory responses by augmenting the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [9]. These cytokines lead to asthmatic pathologic alterations and lung injury [10]. It has been reported that pro-inflammatory cytokines are activated by multiple signaling pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways [11,12]. In addition, an increasing body of evidence shows that TLR4 is an important sensor for LPS [2,13], which activates the adaptor molecules that recruit cytoplasmic proteins to the membrane to induce signaling cascades and subsequently change gene transcription, and promotes inflammation [14]. Therefore, blocking the TLR4-mediated signaling pathways may be a more effective approach to treat ALI. Plantamajoside, a major ingredient isolated from Plantago asiatica L. (Plantaginaceae), has been reported to have many pharmacological activities, such as anti-oxidant, anti-proliferative, anti-viral, diuretic, and anti-inflammatory properties [15–17]. Plantamajoside (C29H36O16), a unique component identified in Herba plantaginis, is a phenylpropanoid glycoside [18]. Its chemical structure is shown in Fig. 1. However, there are few studies that have investigated the effects of plantamajoside on LPS-induced ALI in mice and in RAW264.7 cells. Therefore, we aimed to investigate whether plantamajoside could protect against LPS-induced ALI in mice and in RAW264.7 macrophages and to elucidate the potential anti-inflammatory mechanism that involves the expression of the NF-κB and MAPK signaling pathways. The results of the present study might support a potential clinical application for plantamajoside in treating ALI.

2. Materials and methods

2.1. Reagents

Plantamajoside (purity > 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LPS (Escherichia coli 055:B5) was purchased from Sigma (St. Louis, MO, USA). RAW264.7 cells were purchased from the American
2.2. Animals and treatment

A total of 60 BALB/c mice (6 weeks old, 18–22 g weight) were obtained from the Experimental Animal Center of Wuhan University (Wuhan, China). All of the mice were maintained on a 12 h light/dark cycle for 1 week prior to an experiment and were fed ad libitum. All experiments followed the guidelines for the care and use of laboratory animals published by the NIH. This study was approved by the Huazhong Agricultural University Animal Care and Use Committee.

Mice were randomly classified into six groups to induce the ALI model: control group, LPS group, plantamajoside + LPS groups (25, 50, and 100 mg/kg group), and dexamethasone + LPS group (DEX group). The plantamajoside was dissolved in physiological saline to result in final concentrations of 25, 50, and 100 mg/kg, according to the weight of the mice. LPS was diluted in phosphate buffered saline (PBS) to result in a final concentration of 0.5 μg/μL. The method for creating the LPS-induced ALI model was previously described [19]. Briefly, the mice were intranasally administered 50 μL of LPS to induce ALI. The control group was intranasally administered 50 μL of PBS. At 24 h after perfusion, the plantamajoside groups were intraperitoneally injected with different plantamajoside concentrations (25, 50 or 100 mg/kg) three times at 6, 12, 18 h. The DEX group was given dexamethasone (5 mg/kg) according to the same treatment schedule. The control group was given the same volume of physiological saline. Then, the mice were anesthetized with sodium pentobarbital and killed. The lung tissues were collected and stored at −80 °C until analysis.

2.3. Histopathologic evaluation of lung tissue

Lung tissues were excised and fixed with 10% neutral buffered formalin for 24 h. Subsequently, the tissues were embedded in paraffin, sliced, and then stained with hematoxylin and eosin (H&E). Lastly, pathological changes in the lung tissues were observed under a light microscope.

2.4. Lung wet/dry (W/D) ratio

The lung tissues were collected to calculate the lung wet to dry ratio. The wet lung tissues were separated from mice and the weight was recorded. Subsequently, the lung tissues were incubated at 80 °C in a drying oven for 24 h to obtain the dry weight. Then, the lung W/D ratio was calculated.

2.5. The myeloperoxidase (MPO) assay

The activity of MPO is another measure of phagocytic infiltration of parenchymal tissue. The lungs tissues from each group were collected and homogenated. After centrifugation, the MPO in the supernatant was detected using an assay kit (Jiangcheng company, Nanjing, China) following the manufacturer’s protocol and measured with a spectrophotometry at 460 nm.

2.6. Cell culture and treatment

RAW264.7 cells were cultured in DMEM/F12 medium with 10% fetal bovine serum at 37 °C with 5% CO2 incubation. The cells were pretreated with various concentrations of plantamajoside (25, 50, 100 μg/mL) for 1 h and then were stimulated with LPS (0.5 μg/mL) for 6 h. Cells that were not given any treatment were used as a control.

2.7. Cell viability assay

Cell viability was examined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells (1 x 10^5 cells mL⁻¹) were plated in 96-well plates at 37 °C for 12 h. Then, the cells were treated with the concentrations of plantamajoside (25, 50, 100 μg/mL) for 24 h. Subsequently, 20 μL of MTT (5 mg/mL) was added for 4 h at 37 °C, the supernatant was removed, and 100 μL of dimethylsulfoxide per well was added. The optical density (OD) was read at 570 nm with a microplate reader (Thermo Scientific Multiskan MK3, USA).

2.8. Cytokine determination by ELISA assay

The effects of plantamajoside on the levels of cytokines induced by LPS were determined in lung tissue and in RAW264.7 cells. The lung tissues were collected and homogenized in ice-cold PBS and centrifuged at 10,000 g for 10 min at 4 °C. Then, the supernatants were collected. The supernatant of the cell culture was also harvested. The levels of TNF-α, IL-1β, IL-6, and IL-10 in all supernatants were detected by ELISA according to the manufacturer’s directions. Lastly, the OD was read at 450 nm with a microplate reader (Thermo Scientific Multiskan MK3, USA).

2.9. Quantitative PCR analysis

Total RNA was extracted from lung tissues and RAW264.7 cells by Trizol (Invitrogen, USA) according to the manufacturer’s instructions. The concentration of total RNA was measured at a 260/280 nm ratio, then cDNA was synthesized by reverse transcription. The primers used for determining the levels of mRNA are shown in Table 1. The amplification conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 60 s. The PCR reaction system (20 μL in total) contained 10 μL of SYBR qPCR Mix (Roche, Basel, Swiss), 1 μL of each primer, 1 μL of cDNA, and 7 μL of nuclease-free water. The relative gene expression levels were normalized by GAPDH using the 2^(-ΔΔCT) comparative method.

2.10. Western blotting analysis

Lung samples and cells were dissociated by RIPA lysis buffer supplemented with protease inhibitor and centrifuged at 12,000 g for 15 min at 4 °C. The protein concentrations were determined by using the BCA protein assay kit (Biosharp, China). Then, proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking for 2 h with 5% nonfat dry milk, proteins were hybridized with the
special primary antibody (1:1000 dilution) at 4 °C for 12 h. Then, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (1:4000 dilution) for 1 h at room temperature. The densitometric values of immunoblot signals were developed with the ECL Plus Western Blotting Detection System (ImageQuant LAS 4000 mini, USA).

2.11. Statistical analysis

SPSS 15.0 software was used to analyze the data. The results are presented as the mean ± S.E.M. The differences between groups were analyzed by one-way ANOVA. A p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of plantamajoside on LPS-induced lung injury

The lung tissues were collected, and the pathological sections, W/D ratio, and MPO methods were used to evaluate the lung injury. The histopathological results showed that the control group had a normal alveolar wall structure (Fig. 2A). The LPS group had inflammatory cell infiltration, alveolar congestion, and incrassation in alveolar walls (Fig. 2B). However, in the plantamajoside treatment groups and the DEX group the inflammatory cell infiltration and alveolar congestion were relieved, and the thickness of alveolar walls was reduced (Fig. 2C-F). In Fig. 2G, the W/D ratio result indicated that the LPS group had a large increase in the lung W/D ratio compared with the control group.

Fig. 2. The effects of plantamajoside on LPS-induced lung injury. Histopathological changes in lung tissues after LPS stimulation (HE, ×200). (A) Control group, (B) LPS group, (C) LPS + DEX group, (D-F) Plantamajoside (25, 50, 100 mg/kg) groups; lung W/D ratio (G); MPO assay (H). The data are presented as the mean ± S.E.M. (n = 10). The CG was the control group, the LPS was the LPS-stimulated group, the DEX was the dexamethasone group, the 25, 50 and 100 were plantamajoside administration groups with a dose of 25, 50 and 100 mg/kg, respectively, in the mice. *p < 0.05 vs. the control group, †p < 0.05 vs. the LPS group.
The lung W/D ratio was obviously decreased in the DEX group. However, compared with the LPS group, the lung W/D ratio was decreased in the plantamajoside groups in a dose-dependent manner ($p < 0.05$). A further experiment was carried out on MPO activity to evaluate the effects of plantamajoside on LPS-induced ALI. The results showed that MPO activity in the LPS group was greatly increased compared with the control group ($p < 0.05$). In contrast, the MPO activity was significantly reduced in the DEX group and the plantamajoside groups compared with the LPS group (Fig. 2H).

### 3.2. Effects of plantamajoside on cell viability

The potential cytotoxicity of plantamajoside on RAW 264.7 cells was evaluated using the MTT assay. The results showed that cell viability was not affected by plantamajoside administration (Fig. 3).

### 3.3. Effects of plantamajoside on the levels of cytokines

The expressions of cytokines in lung tissues and in RAW264.7 cells were determined using ELISA and qPCR assays. The results are shown in Fig. 4. In lung tissues and in RAW264.7 cells, ELISA results showed that LPS significantly increased the expression of IL-1β, IL-6 and TNF-α. Plantamajoside and dexamethasone significantly attenuated the expression of IL-1β, IL-6 and TNF-α, but plantamajoside promoted the secretion of IL-10 ($p < 0.05$, as shown in Fig. 4A). qPCR results suggested that LPS significantly increased the levels of IL-1β, IL-6 and TNF-α mRNA compared with the control group. Plantamajoside and dexamethasone inhibited the levels of IL-1β, IL-6 and TNF-α mRNA, but plantamajoside promoted the level of IL-10 mRNA in a dose-dependent manner ($p < 0.05$, as shown in Fig. 4B). These results showed that plantamajoside inhibited the level of pro-inflammatory cytokines induced by LPS and promoted the secretion of IL-10 in lung tissues and in RAW264.7 cells.

### 3.4. Plantamajoside inhibits the expression of TLR4 induced by LPS

TLR4, belonging to the family of TLRs, participates in recognizing exogenous pathogen-associated molecular patterns and endogenous ligands [20]. The effect of plantamajoside on the expressions of TLR4 was determined by qPCR and western blot in lung tissues and RAW264.7 cells. The results showed that the mRNA and protein expressions of TLR4 were increased in the LPS group. The expression of TLR4 was inhibited in the DEX group. Plantamajoside inhibited the LPS-induced TLR4 mRNA and protein expression in a dose-dependent manner in lung tissues and in RAW264.7 cells ($p < 0.05$, as shown in Fig. 5).

### 3.5. Effects of plantamajoside on the NF-κB pathway in LPS-induced ALI

NF-κB is an essential regulator in inflammatory processes [21]. To determine the hindering effect of plantamajoside on the LPS-stimulated NF-κB pathway, NF-κB p65 and IκBα proteins were detected using western blot. In lung tissues, the phosphorylated p65 and IκBα proteins were significantly increased in the LPS group compared with the control group. In contrast, the phosphorylated p65 and IκBα proteins were dose-dependently reduced in the plantamajoside groups. The phosphorylated p65 and IκBα proteins were significantly reduced in the LPS group, but decreased in the plantamajoside treatment groups in a dose-dependent manner ($p < 0.05$, as shown in Fig. 6B).

### 3.6. Effects of plantamajoside on the MAPK pathway in LPS-induced ALI

The MAPK pathway is also a critical mediator in the inflammatory response [22]. To further determine whether MAPKs are affected by plantamajoside, the phosphorylation levels of three MAPKs were measured. In lung tissues, the phosphorylation levels of JNK, p38 and ERK were significantly increased in the LPS group, but decreased in the plantamajoside treatment groups, suggesting the hindering effect of plantamajoside on LPS-stimulated RAW264.7 cells. These results showed that plantamajoside inhibited the phosphorylation of JNK, p38 and ERK. With increasing doses of plantamajoside, the inhibitory effect was gradually prominent ($p < 0.05$, as shown in Fig. 7A). Similar results were also observed in the RAW264.7 cells. In these cells, the phosphorylation levels of JNK, p38 and ERK were significantly decreased in the LPS group, but decreased in the plantamajoside treatment groups in a dose-dependent manner ($p < 0.05$, as shown in Fig. 7B).

### 4. Discussion

Plantamajoside has been reported to produce anti-inflammatory effects [16] but no adverse effects after 90 days of repeated oral administration in rats [23]. In the present study, we observed the effects of plantamajoside on LPS-induced ALI in mice. The results showed that pretreatment with plantamajoside significantly improved the pulmonary histology. Because edema is a typical symptom of inflammation, LPS-induced ALI is characterized by lung edema [24]. To quantify the effects of plantamajoside on lung injury, we first used the lung W/D ratio. The results showed that plantamajoside reduced the lung W/D ratio in a dose-dependent manner. Inflammation of activated neutrophils into the lung is an important constituent of the inflammatory response in ALI [25]. In addition, MPO is a major constituent of neutrophil cytoplasmic granules, and its activity becomes a direct measure of neutrophil sequestration in the tissue [7]. The results showed that the MPO activity induced by LPS was significantly decreased by plantamajoside administration. In addition, the macrophage is an immune cell and often plays a critical role in the inflammatory response [26]; thus, we also detected the effect of plantamajoside on LPS-stimulated RAW264.7 cells. The results of the MTT assay showed that the doses of plantamajoside were not cytotoxic, which was consistent with the results from a previous study [23]. These results demonstrated that plantamajoside had a protective effect in LPS-induced ALI.

A complex network of cytokines, including IL-1β, IL-6 and TNF-α, promote the inflammatory response in LPS-stimulated ALI [27]. IL-1β is a pro-inflammatory cytokine that is released from macrophages upon stimulation with LPS or other inflammatory stimuli [28]. LPS treatment of RAW264.7 macrophage cells significantly enhanced the level of TNF-α and IL-6 [29]. It has been proven that the levels of IL-1β, IL-6 and TNF-α serve as predictive markers in ALI [6]. In the present study, we found that plantamajoside suppressed the expression of these cytokines, suggesting its potential therapeutic efficacy in LPS-induced ALI.

![Fig. 3](image-url) The effects of plantamajoside on cell viability. RAW 264.7 cells were cultured with various concentrations of plantamajoside (0–100 µg/ml) for 24 h. Cell viability was measured using the MTT assay. The values were obtained from three independent experiments and are represented as the mean ± S.E.M. ($n = 10$). The CG was the control group, the LPS was the LPS-stimulated group, the DEX was the dexamethasone group, and the 25, 50 and 100 were the plantamajoside administration groups with a dose of 25, 50 and 100 µg/ml in the cells.
Fig. 4. The effects of plantamajoside on the production of cytokines. A. The expressions of IL-1β, IL-6, TNF-α and IL-10 proteins induced by LPS were detected using ELISA in lung tissues and in RAW 264.7 cells. B. The levels of cytokine IL-1β, IL-6, TNF-α and IL-10 mRNAs induced by LPS were determined by qPCR in lung tissues and in RAW 264.7 cells. GAPDH was used as the control. The data represent the mean ± S.E.M. (n = 10). The CG was the control group, the LPS was the LPS-stimulated group, the DEX was the dexamethasone group, the 25, 50 and 100 were plantamajoside administration groups with a dose of 25, 50 and 100 mg/kg, respectively, in the mice and 25, 50 and 100 μg/mL, respectively, in the cells. *p < 0.05 vs. the control group, †p < 0.05 vs. the LPS group.
of pro-inflammatory cytokines in a dose-dependent manner, and at the same time, plantamajoside also enhanced the level of IL-10. The above results confirmed that plantamajoside might protect against LPS-induced ALI by reducing the transcription and production of these pro-inflammatory cytokines and by promoting the level of IL-10.

To further understand the mechanism by which plantamajoside exerts its anti-inflammatory activity, we then investigated the effects

Fig. 5. The effects of plantamajoside on the expression of TLR4. **A**. The expression of the TLR4 protein was detected using western blot in lung tissues and in RAW 264.7 cells. **B**. The level of TLR4 mRNA was determined by qPCR in lung tissues and in RAW 264.7 cells. GAPDH was used as the control. The data represent the mean ± S.E.M. (n = 10). The CG was the control group, the LPS was the LPS-stimulated group, the DEX was the dexamethasone group, the 25, 50 and 100 were plantamajoside administration groups with a dose of 25, 50 and 100 mg/kg, respectively, in the mice and 25, 50 and 100 μg/mL, respectively, in the cells. *p < 0.05 vs. the control group, †p < 0.05 vs. the LPS group.

Fig. 6. The effects of plantamajoside on the expression of NF-κB pathway activation. **A**. The expression of IκBα and p65 proteins were analyzed using specific antibodies in lung tissues. **B**. The expression of IκBα and p65 proteins in RAW264.7 cells. β-actin was used as the control. The data represent the mean ± S.E.M. (n = 10). The CG was the control group, the LPS was the LPS-stimulated group, the DEX was the dexamethasone group, the 25, 50 and 100 were plantamajoside administration groups with a dose of 25, 50 and 100 mg/kg, respectively, in the mice and 25, 50 and 100 μg/mL, respectively, in the cells. *p < 0.05 vs. the control group, †p < 0.05 vs. the LPS group.
of plantamajoside on LPS-induced activation of the NF-κB and MAPK signaling pathways in lung tissues and RAW264.7 cells. NF-κB, a nuclear transcription factor, is a regulator of inflammatory processes [30]. MAPKs (JNK, p38 and ERK) play important roles in modulating cell growth and differentiation as well as in the control of the cellular responses to cytokines and various stresses [31]. Moreover, it has been reported that NF-κB and MAPK signaling pathways are involved in the LPS-induced model of ALI [11,32]. In the present study, the results showed that plantamajoside obviously inhibited the phosphorylation of NF-κB and MAPKs in LPS-stimulated ALI.

It is well-known that TLR4 stimulated by LPS induces the release of vital pro-inflammatory cytokines which are necessary to activate potent immune responses [14]. Many studies have reported that LPS triggers the immune response via TLR4 and activates the NF-κB and MAPKs signaling pathways [33,34]. Therefore, we also assessed the expression of TLR4 in LPS-induced ALI and in RAW264.7 cells. The results showed that plantamajoside could inhibit the expression of TLR4 in a dose-dependent manner. Our results indicate that plantamajoside suppressed the activation of NF-κB and MAPKs by preventing TLR4-mediated signaling pathways.

In summary, our study suggests that plantamajoside had a protective function in LPS-stimulated ALI in mice. It could suppress the production of pro-inflammatory cytokines by inhibiting the activation of the TLR4-mediated NF-κB and MAPK signaling pathways. In addition, plantamajoside had no side effects on the body. Therefore, it is hoped that plantamajoside may be a potential drug for the treatment of pulmonary inflammation.

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References


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